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(57) Abstract

The invention relates to cytidine deaminase and its enhancers for use in inhibiting cell proliferation, especially haemopoiesis and to the use of cytidine deaminase inhibitors to stimulate cell proliferation, especially in leukopenic conditions and to mobilize stem cells to the blood.

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CELL GROWTH REGULATORS

The present invention is concerned with the control of cell proliferation, especially the control of haemopoiesis and/or granulopoiesis. In particular the present invention is concerned with the use of cytidine deaminase and/or its regulators to control cell proliferation.

The mammalian body contains cells having enormously diverse structures and functions, and the mechanisms of differentiation and development have been the focus of much study. It is known that for systems of cells having a continuous turnover the mechanism commonly involves a reservoir of pluripotent stem cells which divide and constantly supply new cells to the system. While initially homogeneous the stem cells supplied from the "reservoir" soon become committed to one or other morphology and subsequently develop into the required functional cells.

Examples of such stem cell systems are the haemopoietic system in bone marrow and the epithelial and epidermal systems.

It is already known that mature granulocytes and granulocyte extract (GRE) affect granulopoiesis (see Rytömaa et al., p 106, "Control of cellular growth in adult organisms" Academic Press (London), 1967 ed. Teir and Rytömaa). Interestingly both stimulating and inhibitory effects have been noted (see, for example, Bøyum et al, Eur J. Haematol 38: 318 (1987) and Helgestad et al, Acta Physiol Scand 133: 41 (1988)). However, it has been suggested that the results obtained in vitro depend upon the experimental design adopted

(see Helgestad et al, supra).

Regulation of cell proliferation by stimulation or inhibition continues to be of interest as a treatment for diseases or conditions where natural control of cell division has malfunctioned, for example in cancer (in particular leukemia) or AIDS.

The enzyme cytidine deaminase (CDD) is responsible for the conversion of cytosine to uracil in mammalian cells. This reaction is also catalysed by other enzymes, including deoxycytidylate deaminase and a sequence specific cytidine deaminase involved in the editing of apolipoprotein B mRNA.

CDD has been isolated from human tissues, and sources include the liver, spleen and placenta (see Ho, Cancer Res 33: 2816-2820 (1973) and Cacciamani et al, Arch Biochem Biophys 290: 285 (1991)). Additionally, Chabner et al. (J Clin Invest 53: 922-931 (1974)) reported the partial purification and characterisation of CDD from normal and leukemic granulocytes. A highly purified form of CDD (E.C. 3.5.4.5) has been isolated from human placenta (see Laliberté et al, Cancer Chemother Pharmacol 30: 7-11 (1992)).

CDD is known to be a 52 kD protein composed of four subunits which are currently thought to be identical. CDD continues to be of interest in the field of cancer therapy since this enzyme is responsible for the deamination of the widely used anti-cancer agent cytidine arabinoside (Ara-C). The product of this deamination reaction is uridine arabinoside (Ara-U) which, besides being much less effective therapeutically than Ara-C, is thought to cause neurotoxicity. Since in Ara-C chemotherapy the active agent is continually degraded by cytidine deaminase, high dosages of Ara-C are often administered in order to maintain that

compound at a therapeutically effective level in the body. However central nervous system toxicity including cerebellar dysfunction and peripheral neuropathy have been observed in patients treated with high dosages of Ara-C. To minimise the amount of Ara-C required to maintain a therapeutically effective level, the CDD inhibitor tetrahydrouridine (THU) has been administered as part of the chemotherapy regime (see Kreis et al, Leukemia 5(11): 991-998 (1991)). THU acts by competitively inhibiting CDD, thus reducing the amount of Ara-C which is deaminated by that enzyme. Other inhibitors of CDD such as Zebularine, 5-F-Zebularine and diazepinone riboside have also been suggested for use in combination chemotherapy with Ara-C or its analogue 5-aza-2'-deoxycytidine (see Laliberté et al, Cancer Chemother Pharmacol 30: 7-11 (1992)). In each of the studies discussed above, administration of CDD inhibitors was only proposed as a supplement, to prevent degradation of the agent causing inhibition of cell proliferation, namely Ara-C and 5-aza-2'-deoxycytidine.

It has now been found that cytidine deaminase (CDD) produced for example by mature granulocytes acts directly to inhibit the proliferation and colony formation of human and murine granulocyte-macrophage progenitor cells (GM-CFC).

The present invention thus provides the enzyme cytidine deaminase (CDD) or a functional fragment thereof for use in the regulation of cell proliferation, particularly in the regulation of haemopoiesis, for example in the inhibition of haematopoietic stem cell proliferation or of granulopoiesis. Thus, for example, it may be desirable to protect haematopoietic stem cells during anti-cancer chemotherapy or radiotherapy by interrupting their cell division cycle.

Compositions comprising cytidine deaminase form a further aspect of the present invention.

Preferably, the cytidine deaminase is in an at least partially purified form. Substantially purified cytidine deaminase is also preferred.

Generally, the effect of cytidine deaminase is inhibitory although the degree of inhibition depends on the concentration present. In particular, a reduced inhibitory effect has been observed at high concentrations of CDD ie. a bell-shaped dose-response curve is obtained.

The cytidine deaminase used may be derived from any convenient source. Thus, for example, the enzyme may be isolated from bodily organs such as the liver or spleen (see Ho (1973), Chabner (1974) and Cacciamani (1991) supra). More conveniently, however, the enzyme may be isolated from cell cultures - the cells either producing CDD naturally or as a result of transformation with recombinant DNA. In a particularly preferred embodiment the enzyme CDD is produced from cells grown in culture which have been transformed or transfected with a DNA vector coding for cytidine deaminase, the cytidine deaminase gene being controlled by appropriate promoter and/or regulator sequences.

Harvesting and purification of cytidine deaminase may be by any suitable method. Suitable purification and separation techniques are well-known to those skilled in the art and include centrifugation, precipitation, dialysis, chromatography, including affinity chromatography and column chromatography.

Human granulocytes contain large amounts of CDD, 9-10 times more than mononuclear cells. The amount drops to 36% of the normal value in the granulocytes with chronic myelogen leukemia. The reduction per cell is

even greater with acute myelogen leukemia. Recent findings indicate that the amount of CDD in normal and leukemic cells can be approximately equivalent, however, CDD activity is reduced in the leukemic cells.

The high number of leukemic cells can thus be explained by a reduction of CDD or active peptide groups from CDD. CDD may therefore be used in the treatment of the symptoms.

Thymidine is required as a co-factor for CDD as has been previously observed for crude granulocyte extract (see Helgestad et al, supra and Bøyum et al, Eur J. Haematol 40: 119 (1988)). In agar cultures with human granulocyte cells the inhibitory effect of CDD was detectable with a thymidine concentration of 10^{-6}M.

Thus, the present invention also provides a combination of cytidine deaminase or a functional fragment thereof and one or more cofactors which enhance the activity of cytidine deaminase, for use in the regulation of cell growth, in particular haemopoiesis, especially granulopoiesis, for example in chronic myelogen leukaemia. Such cofactors include nucleosides or analogues thereof, in particular pyrimidine nucleosides and analogues thereof, especially those selected from the list consisting of thymidine, deoxycytidine, deoxyuridine and their phosphate derivatives. Thymidine is especially preferred. Conveniently the thymidine is present at a concentration range of $5x10^{-3}$ to $1x10^{-6}$M. The thymidine or other cofactor may be administered simultaneously or sequentially with the CDD.

Thymidine is of course naturally present in the body and it may therefore not be essential for thymidine to be administered at all for inhibition of cell division to be achieved.

Ensminger et al (Cancer Res 37: 1857 (1977)) reported a median thymidine concentration of 0.2 x 10^{-6}M in human plasma. Whilst this concentration appears lower than that required in vitro, it is believed that thymidine concentration at particular sites in the body, for example in the bone marrow, may be locally elevated. In the bone marrow for example large numbers of erythroid nuclei are extruded from normoblasts and then engulfed by macrophages in which DNA degradation takes place, thus generating inter alia thymidine.

Thymidine has itself been reported to cause inhibition of cell proliferation (see Blumenreich et al, in Cancer Research 44: 2203-2207 (1984), Chiuten et al, in Cancer Research 40: 818-822 (1980) and Leyva et al, in J. Cancer Res. Clin. Oncol. 107: 211-216 (1984)). Whilst relatively high dosages of thymidine (giving millimolar concentrations in the plasma) did cause some remission in cancer patients, it was not shown to be an effective anti-cancer therapy and induced side-effects such as nausea, vomiting and hepatotoxicity. The inhibitory effect of thymidine on cell division was believed to be due to allosteric inhibition of the enzyme ribonucleotide reductase.

Whilst we do not wish to be bound by theoretical considerations, several mechanisms can be put forward to explain the inhibitory effect of CDD. One possibility is that the combination of CDD and thymidine have a synergistic effect on the depletion of the deoxycytidine precursors required for DNA synthesis. Thymidine may activate CDD directly or may neutralize an antagonist of CDD. Previous studies have indicated that excess thymidine (alone) may cause increased levels of deoxythymidine triphosphate (dTTP) which is believed to inhibit ribonucleotide reductase (see Reichard,

Biochemistry 26: 3245 (1987) and O'Dwyer et al, Cancer Res 47: 3911 (1987)). Inhibition of ribonucleotide reductase would prevent or reduce the conversion of cytidine to deoxycytidine. As indicated above CDD deaminates deoxycytidine, again lowering the concentration of this metabolite. Whilst thymidine and CDD separately only have a marginal effect in isolation, their combination could cause a significant deficiency in deoxycytidine triphosphate (dCTP) (which is one of the precursors of DNA) leading to failure of cells to divide.

In an alternative hypothesis CDD binds to a specific receptor on the cell surface, the binding or subsequent steps in the mechanism being enhanced by the co-factor thymidine. The binding of CDD to its receptor may then cause transmission of a signal which prevents or inhibits cell division.

Thymidine thus acts to enhance the effect of cytidine deaminase and at higher concentrations, for example 3-6 x 10^{-5}M, thymidine causes CDD to exhibit a much stronger inhibitory effect ie. 50-90% suppression of normal cellular proliferation. Other regulators which enhance the CDD-mediated inhibition of cellular proliferation include the nucleosides deoxycytidine and deoxyuridine. Both deoxycytidine and deoxyuridine may replace thymidine in enhancing the inhibitory action of CDD. Additionally, a mixture of the nucleosides deoxycytidine and thymidine act synergistically in promoting the inhibition of cell division exhibited by CDD. Thymidine phosphates such as thymidine monophosphate and thymidine triphosphate act in the same way as thymidine. These are inhibitors of deoxycytidine monophosphate deaminase (dCMPD) and other inhibitors of dCMPD such as 5-fluorodeoxyuridine (5-FdU) and 5-fluoro-

deoxyuridine monophosphate (F-dUMP) act similarly.

CDD and its potentiators could be used in combination therapy with anti-cancer agents. By use of CDD and CDD potentiators, cells of interest, for example the bone marrow, could be taken out of cycle and thus be protected (ie. made less sensitive) to the effects of anti-cancer agents.

In our experiments thymidine was used at rather low concentrations, but nevertheless yielded 20-30% inhibition ($p<0.01$) ($4-8\times10^{-5}$ M thymidine) in combination with CDD in a granulocyte extract (GRE) from 1.6×10^{5} granulocytes (/ml). At the high GRE concentration (10^{6} cells/ml) thymidine (8×10^{-5}M) yielded no inhibition. FdUMP had itself an antiproliferative effect at the highest concentrations, but this effect was partly abolished when combined with thymidine. The most striking effect of FdUMP was the ability to induce inhibition also together with a high GRE concentration.

In further experiments, mononuclear human blood cells were cultured in fetal calf serum or serum from the same individuals (autologous serum), at two different thymidine concentrations. In comparison with fetal calf serum cultures there was a striking effect on colony formation by human serum in cultures with 3.3×10^{-5} M thymidine, the colony number was reduced by 50%, and with 16×10^{-5} M a 90% reduction was observed. The effect of thymidine was to a large extent prevented by adding THU to the cultures. The thymidine increase had no effect on colony formation in fetal calf serum cultures but addition of GRE (containing CDD) caused a strong inhibition, which could be abrogated by THU. It thus appears that human serum contains CDD that induces inhibition of colony formation when thymidine is added. Accordingly it was shown by thin layer chromatography

that CDD activity could easily be detected in fresh human serum. No appreciable activity was found in fetal calf serum. However these sera have been subjected to freezing/thawing which tends to reduce CDD activity. In any case, these finding may suggest the CDD plays a physiological role in regulation of white cell production.

It follows from the above considerations that THU may be used in the treatment of some diseases where there is a need for stimulating cell proliferation by inactivating CDD. Furthermore thymidine and other CDD enhancers may be used in certain diseases to enhance the inhibitory effect of CDD in tissue fluid. Alternatively thymidine and CDD may be used in combination. Large doses of thymidine have been used in the treatment of malignant diseases, with little success. However, for a disease like chronic granulocytic leukemia, thymidine dosage may enhance the inhibitory effect of CDD whereby production of granulocytes may be maintained at a lower level.

The invention thus provides CDD potentiating nucleosides or analogues thereof for use in the regulation of cell proliferation mediated by cytidine deaminase, for example chronic myelogen leukaemia. Thymidine, deoxycytidine and deoxyuridine are examples of suitable nucleosides. They may be used in the form of their phosphate derivatives. Thymidine or a combination of thymidine and deoxycytidine are preferred. The nucleosides or analogues thereof are conveniently added to give concentrations of 10^{-6} to 10^{-4}M, for example 5×10^{-4} to 5×10^{-5}M.

Inhibitors of CDD itself, for example the inhibitor THU discussed above, may also be used according to the invention to control or prevent the inhibition of cell

proliferation. In particular, inhibition of CDD may be used to control or prevent the inhibition of cell proliferation due to CDD. Other inhibitors of CDD which affect cellular proliferation in the same way include Zebulaire, 5-F-Zebulaire, 5-chloromercuricytidine, CV6, 2-azido-2-deoxycytidine, 5,6-didehydro-uridine and diazepinone riboside. Additionally antibodies to CDD may also be used as CDD inhibitors where these bind to or interfere with the active site of CDD. As discussed above, the use of THU with the anti-leukemic drug Ara-C has already been investigated (see Kreis et al, supra). Whilst the action of THU in competitively inhibiting CDD was appreciated in that study, THU was administered simply to prevent the undesirable deamination of the drug of interest (Ara-C) by CDD. It was, however, not appreciated that CDD affected cell proliferation itself.

CDD inhibitors may be of use in the regulation of leukopenic situations in general and more particularly after anti-cancer treatment or bone marrow transplantations or in connection with the treatment of infections. CDD inhibitors are believed to increase the number of white blood cells and/or their activity.

It will be appreciated that dCMPD referred to above is antagonist of CDD and thus acts as a CDD inhibitor.

In a further aspect, the present invention thus provides the use of CDD inhibitors in the regulation of cell proliferation, in particular cell proliferation mediated by CDD. Another aspect of the invention provides the use of CDD inhibitors, such as THU, in the manufacture of a medicament for the regulation of cell proliferation, in particular cell proliferation mediated by CDD. In a still further aspect, the present invention provides the use of one or more CDD inhibitors in the preparation of a medicament for the mobilization

of haematopoietic stem cells to the blood.

In a further aspect, the present invention provides a pharmaceutical composition comprising (a) cytidine deaminase or a functional fragment thereof optionally in combination with a CDD potentiating nucleoside or analogue thereof or (b) an inhibitor of cytidine deaminase.

In the pharmaceutical compositions according to the present invention the usual pharmaceutically acceptable inert carriers, diluents, additives, flavourings and/or colourings may of course be present as required. Suitable adjuvants and excipients will be known to those skilled in the art.

In a still further aspect, the present invention provides a method of treatment of the human or non-human animal body to regulate cell proliferation, said method comprising administering to said body cytidine deaminase or a functional fragment thereof. The cytidine deaminase or functional fragment thereof may also be used in combination with one or more cofactors which enhance the activity of cytidine deaminase. A further method of treatment to regulate cell proliferation which is provided by the present invention comprises administration of one or more cytidine deaminase inhibitors.

In the diagrams:

Fig. 1 illustrates the effect of different concentrations of deoxycytidine, alone or in combination with thymidine in inhibiting colony formation in the presence of granulocyte extract (GRE);

Fig. 2 shows how colony formation is affected by addition of GRE, with and without thymidine or deoxyuridine;

Fig. 3 depicts the relationship between colony number and dose of GRE at different concentrations of thymidine;

Fig. 4 shows the inhibition of colony formation at varying concentrations for different Mono Q fractions of GRE;

Fig. 5 is a bar chart relating to the dose of GRE to colony number, optionally including Ara-C at concentrations of 10^{-7}M and 10^{-5}M;

Fig. 6 is a bar chart showing how colony number is affected by the concentration of azacytidine with and without GRE;

Fig. 7 illustrates how colonies of mouse BMC and human blood cells treated with GRE and affected at different concentrations of THU:

Fig. 8 shows the inhibition of colony formation and CDD activity (measured by deoxyuridine

concentration) for different fractions of GRE following Mono Q;

Fig. 9 illustrates the results of MTT-assay for different Mono Q fractions of GRE; and

Fig. 10 shows the results of MTT-assay for fraction 5 (of the Mono Q purification of GRE) at different concentrations.

The present invention is further illustrated by the following, non-limiting, examples.

Materials and Methods

Chemicals: Thymidine, cytidine, deoxycytidine, deoxyuridine, 5-azacytidine, 5-aza-2'-deoxycytidine, and cytosine arabinoside were obtained from Sigma. Tetrahydrouridine was obtained from Calbiochemic (La Jolla, Ca). Deoxy[5-^{3}H]-cytidine, specific activity 1.07 TBq/mmol, was obtained from Amersham. McCoy's 5A medium, RPMI 1640 and CMRL 1066 medium were obtained from Flow. The CMRL 1066 medium was used with additives (Helgestad et al, supra). Lymphoprep was provided by Nycomed AS, Oslo. Murine interleukin 3 was obtained from Genzyme (Cambridge, Ma) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF) from Pepro Tech Inc. (Rocky Hill, NJ).

Cells: Bone marrow cells (BMC) were obtained from the femurs of female B_6D_2 mice (Bomholdt gaard, Denmark). Mononuclear cells and granulocytes from human blood and buffy coat samples were separated with Lymphoprep (Bøyum et al, Scand J. Immuno 34: 697 (1991)). A slow centrifugation (60g, 10 min) was included to remove platelets from the mononuclear cells. Contaminating erythrocytes in the granulocyte fractions were lysed by incubating the cells in 0.83% NH_4Cl for 7 minutes at room temperature. After centrifugation (600 g, 7 min), the supernatant was removed, the cells resuspended in 0.9% NaCl and counted. NFS 60 cells, an early murine myeloid cell line, the C6 cell line, a fibroblast type murine cell, and a human bladder carcinoma cell line (5637) were kindly provided by Dr. Andrew King, SmithKline

Beechham (Pa, USA). These cells, and mouse L-cells (NCTC Clone 929), were cultured in RPMI 1640 medium with 10% FCS, and were subcultured once or twice weekly. The supernatant from L-cells was used as source of macrophage CSF. The medium conditioned by the carcinoma cell line (CM 5637) is a rich source of human G-CSF (Welte et al, PNAS 82: 1526 (1985)). The NFS 60 cells were cultured in the presence of 2% (v/v) CM 5637.

Granulocyte extract (GRE): The granulocyte pellet obtained after NH_4Cl treatment and washing was suspended in water for 4-5 minutes, at a concentration of 200 x 10^6 cells/ml. The supernatant was collected after centrifugation and stored at -20°C until used.

GM-CFC assay: Mouse BMC (5 x 10^4 per plate) were cultured in 0.3% agar (Bacto-agar, Difco) in CMRL 1066 medium and 16% fetal calf serum. CM 5637 was used (0.1 ml per 1 ml culture plate) as stimulator. After 7 days of incubation at 37°C and with 7.5% CO_2 in humidified air, the colonies (>50 cells) were counted. Mononuclear human blood cells (5 x 10^5/ml) were cultured for 14 days in 0.3% agar, in McCoys's 5A or CMRL 1066 medum, with 16% FCS. No stimulator was added. Aggregates of more than 40 cells were counted as colonies. The cultures were run in triplicate.

MTT-assay: Proliferation of established cell lines was measured by a colorimetric method (Mosmann, J Immunol Meth 65: 55 (1983)). The cells were seeded in microtitre plates (Costar 3596, Cambridge, Mass.) at a concentration of 2 x 10^4 cells/well in 100 µl of CMRL 1066 medium with 15 x 10^{-5}M thymidine and 5% FCS (Hyclone, Logan, Utah). Fifty µl of test samples in

various dilutions was added to each well. After incubation for 4 days in a humidified atmosphere of 5% CO_2 in air at 37°C, 15 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Tiazolyl Blue) (Sigma) at a concentration of 5 mg/ml in phosphate buffered saline was added, and the cultures were further incubated for 3 hours. Thereafter 100 µl of isopropanol was added to all wells. The microtiter plates were shaken at room temperature for 30 minutes and then the remaining dark blue crystals were dissolved by pipetting. The optical densities of the wells were read on a Dynatec MR 700 microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Cytidine deaminase assays: Reaction mixtures contained 50 mM Hepes, pH 7.5; 0.1 mM deoxy[5-^{3}H]-cytidine (specific activity 18.5 MBq/mmol); and protein fractions to a final volume of 5 µl, and was incubated for 30 minutes at 37°C. The reaction was stopped by addition of unlabelled deoxycytidine and deoxyuridine in equimolar amounts (1 µl of 5 mM each) followed by application on a Polyethyleneimine thin layer sheet (plastic backing, Schleicher & Schüll). The chromatograms were developed in isopropanol/0.1M HCl (7:2) which gives Rf values of 0.73 for deoxyuridine and 0.51 for deoxycytidine, respectively. Spots containing deoxyuridine and deoxycytidine were visualized by UV (254 nm), and radioactivity in each spot determined by scintillation counting.

Protein chromatography: GRE (4 ml, approximately 40 mg protein) was diluted with 6 ml buffer A (50 mM NaCl; 50 mM Hepes, pH 7.5; 1 mM EDTA) and applied to a DEAE-

Sephacel (Pharmacia) column (1.3 x 2.6 cm) preequilibrated with buffer A. The column was washed with 10 ml of buffer A and eluted with 6 ml of 400 mM NaCl (50 M Hepes, 1 mM EDTA). The eluate was subjected to buffer exchange to buffer A with a PD-10 column (Pharmacia), and applied to a Mono Q anion exchange column (0.7 x 5.5 cm, FPLC system, Pharmacia). The column was washed with 20 ml of buffer A and eluted with a linear gradient to 1000 mM NaCl at a flow rate of 0.25 ml/min. Fractions (1 ml) were sterile filtered and tested for inhibitory activity in the GM-CFC assay or for cytidine deaminase activity as indicated above. Active fractions were further purified by gel filtration (1.5 x 25 cm Ultrogel AcA 34) and eluted with buffer B (400 mM KCl; 50 mM Hepes, pH 7.5; 1 mM EDTA, 25% glycerol, 1 mM mercaptoethanol). Fractions (1 ml) were sterile filtered and assayed for GM-CFC inhibitor and cytidine deaminase activity as described above. The gel column was precalibrated with myoglobin (17.5 kD), ovalbumin (47 kD), and blue dextran (reflecting void volume) under identical flow conditions.

Statistics: Students t-test or Wilcoxon's non-parametric test was used for statistical calculations.

EXAMPLE 1

Inhibition of granulocyte-macrophage colony-forming cells (GM-CFC) from human blood

The separate and combined effect of thymidine (3 x 10^{-5} M) and deoxycytidine on the capacity of extract (GRE) from 10^6 granulocytes to inhibit growth of granulocyte-macrophage colony forming cells (GM-CFC) from human

blood were investigated. Mononuclear human blood cells (5×10^5) were cultured in McCoy's medium in agar, and the colonies were counted on day 14. Control cultures contained an average of 30 (18-58) colonies. Mean values (± SD) from 5 experiments are given as a percentage of control value. The cultures were run in triplicate in each experiment. Thymidine or deoxycytidine alone had no apparent effect on colony number. The results are shown in Figure 1.

As illustrated in Figure 1 thymidine and deoxycytidine had an additive effect ($p<0.05$). It is also shown that deoxycytidine could replace thymidine as a co-factor for the granulocyte extract at a concentration of 2×10^{-4}M ie. at a 10x higher concentration than the concentration of thymidine required to produce strong inhibition.

EXAMPLE 2

Inhibition of GM-CFC by thymidine or deoxyuridine with and without granulocyte extract

Human blood GM-CFC were grown in agar cultures. Thymidine or deoxyuridine, alone or with 10^6 GRE (granulocyte extract) were added in McCoy's medium. The inhibitory effect on GM-CFC was observed. McCoy's medium is nucleoside free. A comparison with CMRL 1066 medium (contains 4.0×10^{-5}M thymidine) was also performed.

The results, shown in Figure 2, are the mean values (± SD) from 3 experiments. Figure 2 shows that deoxyuridine can replace thymidine as a co-factor for the granulocyte extract at an approximately 10 fold

higher concentration than thymidine to achieve the same cellular inhibition (5 x 10^{-4}M of deoxyuridine required compared to 10^{-5}M thymidine).

High concentrations (1-5 x 1^{-4}M) of deoxyuridine showed no suppressive affect on GM-CFC. In contrast, the lowest deoxyuridine concentration (10^{-4}) enhanced colony formation ($p<0.01$).

On the other hand, thymidine at high concentration (5 x 10^{-4}M) exerted an inhibitory effect in the absence of granulocyte extract.

EXAMPLE 3

Inhibition of Mouse Bone Marrow cells by GRE

Mouse bone marrow cells (BMC) were cultured for 7 days in CMRL 1066 medium in agar. The supernatant from cultures of the 5637 cell line was used as stimulator. Granulocyte extract was added in increasing doses. Mean values (±SD) were obtained from 3 replicate cultures.

Mouse bone marrow cells have previously been shown to be sensitive to GRE and thymidine (See Bøyum et al, Eur J. Haematol 40: 119 (1988)). Here, it was found that 4 x 10^{-5}M thymidine was sufficient to cause strong (approximately 80%) inhibition of cells cultured in methylcellulose.

To obtain the same degree of inhibition in agar culture, it was found in a comparative study that the thymidine concentration had to be increased by 3-4 fold. The results shown in Figure 3 indicate that strong

inhibition of murine GM-CFC in agar required a thymidine concentration of 13 x 10^{-5}M.

EXAMPLE 4

The effect of GRE on colony formation induced by different stimulators

The low density fraction of mouse BMC separated with Lymphoprep were cultured in McCoy's medium with 13x10^{-5}M thymidine. Recombinant IL-3 (100 units/ml) rGM-CSF (10 ng/ml), L-CSF (0.1 ml/plate) and CM 5637 (0.1 ml/plate) were used as stimulators. Mean values (± SD) from 2 separate experiments.

Table 1

Stimulator	Colony number in control per 5x10^4 BMC	Colony number as a percentage of control value		
		GRE	Thymidine	Thymidine + GRE
IL-3	163±27	86±3	89±3	11±6
GM-CSP	108±16	95±6	115±5	60±7
L-CSF	222±17	100±9	108±10	52±7
CM 5637	262±36	101±6	92±5	30±3

EXAMPLE 5

Isolation of the fraction of GRE with GM-CFC inhibitory activity

Four ml GRE (from $800x10^6$ cells, approximately 40 mg protein) was separated with DEAE-Sephacel and anionic exchange chromatography (Mono Q, see "Materials and Methods") and tested for inhibitory activity on GM-CFC in mouse bone marrow cultured in CMRL 1066 medium in agar with $13x10^{-5}$M thymidine. Fraction 1 indicates the start of elution with 1000 mM NaCl. The elution was completed in 30 minutes. The mean values (± SD) from 10 separations are given in Figure 4.

After anionic exchange chromatography, it was observed that the inhibitory activity of fractions 4 and 5 was lower at the highest concentration (dilution of fraction by 1/50). The phenomenon was not observed for fraction 6, but has been previously reported for crude granulocyte extract (see Bøyum et al (1980) supra).

EXAMPLE 6

Prevention of Inhibitory Action of Cytosine Arabinoside (Ara-C) and Azacytidine by GRE

GM-CFC from human blood were cultivated and the inhibitory effects of GRE, Ara-C and GRE with Ara-C were investigated. The results are shown in Figure 5.

Parallel experiments were performed using azacytidine instead of Ara-C and the results are given in Figure 6.

At concentrations of 10^{-7}M, Ara-C caused almost complete suppression of cell division in GM-CFC. This suppression was alleviated by addition of extract from 10^5 granulocytes. Control experiments showed that GRE from the same batch suppressed GM-CFC in thymidine supplemented cultures (CMRL 1066).

Similarly, crude GRE prevented the inhibitory effect of azacytidine - see Figure 6, ($p<0.01$).

EXAMPLE 7

The effect of tetrahydrouridine (THU) on GRE induced suppression of colony formation by mouse bone marrow cells and human blood cells

The cells were cultured in agar in CMRL 1066 medium with either 3.3×10^{-5}M (human cells) or 13×10^{-5}M (mouse cells) thymidine. GRE from 2×10^5 cells were added per culture plate.

Figure 7 gives the results with mean values (±SD) from 4 mouse cell experiments and 3 human cell experiments.

The inhibitory effect of crude GRE on human and murine GM-CFC was completely abolished by 4×10^{-6}M THU. THU is known to be a specific inhibitor of CDD and these results therefore indicate that CDD is responsible for the inhibitory effect observed. THU itself had no noticeable effect on colony formation.

EXAMPLE 8

Investigation of CDD activity in GRE fraction

The presence of CDD in GRE was investigated directly, by testing for the ability to convert deoxycytidine to deoxyuridine. The active fractions of the Mono Q separation and separation (see Example 5) were further separated by gel filtration. Figure 8 shows the elution profile after gel filtration of cytidine deaminase and inhibitory activity in GRE. Inhibitory activity on mouse GM-CFC was measured in the same fractions. The most active fractions were reexamined for CDD and inhibitory activity, with almost identical results. Closed symbols denote experiments with tetrahydrouridine (THU). The elution positions of molecular weight standards are indicated.

The growth inhibitor and CDD activity were found to copurify, both eluting in a single peak of M_r 50 kD which corresponds to the molecular weight of human CDD reported earlier by Cacciamani et al in Arch Biochem Biophys 290: 285 (1991).

THU at a concentration of 4×10^{-5}M neutralized the inhibitory and deaminating ability in all active fractions.

EXAMPLE 9

Inhibition of mouse GM-CFC, NFS 60 and C6 by the Mono Q fractions of GRE

NFS 60 and C6 cells were cultured in microtitre plates

(20,000 cells/well) for four days and the cell growth was measured with a colorimetric method (MTT). GM-CFC was monitored with the agar colony assay. Fractions obtained after anionic exchange chromatography (Mono Q) were added to NFS 60 and GM-CFC cultures after 800 fold final dilution in growth medium and to the C6 cells after 50 fold dilution. Figures 9 gives the results from the mean (±SD) of triplicate cultures from one representative experiment. The results were confirmed twice, in one experiment with a different set of GRE fractions.

It was found that the inhibitory activity for colony formation affected murine GM-CFC and myeloid cell line NFS 60 to a similar extent. Inhibition of colony formation for the NFS 60 cell line was abolished by THU addition (results not shown).

In contrast, the proliferation of a fibroblast cell line, C6, was not suppressed by the GRE fractions, indicating that there may be some cell specificity for CDD.

EXAMPLE 10

Dose-Response Curve for Fraction 5 from Mono Q purification

The most active fraction from Example 9 (Fraction 5 - see Figure 9) was tested at several concentrations for its ability to inhibit cell replication for murine GM-CFC, NFS 60 and C6 cells. NFS 60 cells and C6 cells were cultured in microtiter plates (20,000 cells/well) for four days and the cell growth was measured with the MTT assay. GM-CFC was monitored with the agar colony

assay. Figure 10 gives the results of mean values (± SD) of triplicate cultures from one representative experiment. The results were confirmed in two additional experiments.

Figure 10 shows the dose-response curve for fraction 5 at different dilutions. The inhibitory effect on GM-CFC and NFS 60 cells was partially alleviated at high concentrations of Fraction 5 GRE, thus giving a bell-shaped response curve.

EXAMPLE 11

Bone marrow cells were cultured for 7 days in McCoy's medium and then the colonies were counted.
Two granulocyte extracts were prepared, the first from $1.6x10^5$ granulocytes per ml and the second from $8x10^6$ granulocytes per ml.

The effect of thymidine and/or 5-fluoro-2-deoxyuridine-monophosphate (FdUMP) alone or together with one of the two granulocyte extracts on proliferation of the colonies was tested. The results are shown in Table 2. When thymidine and FdUMP were combined, the concentration of thymidine was kept constant at $0.8x10^{-4}$ M and FdUMP concentration was varied as shown in Table 2.

Table 2

The effect of thymidine (T), 5-fluoro-2-deoxyuridine-monophosphate (FdUMP) and T+FdUMP on the inhibitory effect of granulocyte extract (GRE) on granulocyte/macrophage colony formation.

Colony number as a percentage of control

Conc^{n} T or FdUMP (M)	T	T + GRE 1	T + GRE 2	FdUMP	FdUMP + GRE 1	FdUMP + GRE 2	T + FdUMP	T + FdUMP + GRE 1	T + FdUMP + GRE 2
0	95	102	90						
$0.8x10^{-4}$	108	79	91	7	0	0	64	0	0
$4x10^{-5}$	109	82	94	50	0	0	76	0	0
$0.8x10^{-5}$	102	95	95	102	59	0	104	70	1
$0.8x10^{-6}$	109	93	80	115	94	63	95	84	62
$0.8x10^{-7}$	104	94	96	100	85	64	107	64	58

GRE 1 = extract from $1.6x10^{5}$ granulocytes/ml. GRE 2 = extract from $8x10^{6}$ granulocytes/ml. When T and FdUMP were combined, the concentration of T was kept constant ($0.8x10^{-4}$ M), and FdUMP concentration was varied as indicated in the left column.

Despite the low concentration of $4\text{-}8x10^{-5}$ M, thymidine caused 20-30% inhibition ($p<0.01$) with GRE 1. With the higher concentration of GRE (GRE 2), thymidine caused less inhibition. FdUMP itself had an antiproliferative effect at high concentrations, but this effect was

abolished when thymidine was also present.

FdUMP together with GRE 2 (high granulocyte extract concentration) caused significant inhibition of cell proliferation.

EXAMPLE 12

Mononuclear human blood cells were cultured in fetal calf serum (FCS) or autologous serum from the donor of the blood cells. The cells were cultured at two different thymidine concentrations ($3.3x10^{-5}$ M thymidine or $16x10^{-5}$ thymidine). Aggregates with more than 40 cells were counted as colonies. THU and/or GRE were added and the effect on colony number was evaluated as a percentage of the control values.

The cultures were run in triplicate and Table 3 shows mean values (± SD) from two experiments. The mean colony number in FCS cultures used as control was 259 ± 61.

Table 3

Colony formation by human blood cells in cultures with fetal calf serum (FCS) or autologous serum. The effect of thymidine, granulocyte extract (GRE) and tetrahydrouridine (THU).

Colony number as a percentage of control values with FCS and low thymidine concentration.

Thymidine Conc. (M)	Serum	Controls	THU	GRE	GRE + THU
$3.3x10^{-5}$	FCS	100 ± 6	100 ± 6	34 ± 7	99 ± 7
$3.3x10^{-5}$	aut.ser	47 ± 6	81 ± 13	28 ± 2	73 ± 12
$16x10^{-5}$	FCS	103 ± 5	96 ± 7	16 ± 7	92 ± 9
$16x10^{-5}$	aut.ser	11 ± 3	63 ± 8	7 ± 4	77 ± 11

The higher thymidine concentration had no effect on FCS cultures. In both culture types addition of GRE from 2 x 10^5 cells per plate caused strong inhibition on colony formation. This was, however reversed by addition of THU.

EXAMPLE 13

The effect of tetrahydrouridine (THU) on haemopoietic progenitor cells in the blood was examined in vivo, in mice.

A dose of 0.6 mg per mouse (strain DBA) was injected intraperitoneally at time -4 hours (THUx1) or time -20 and -4 hours (THUx2), and blood was collected by heart puncture at time 0 hours. White blood cell number was determined and $1x10^5$ cells were seeded in 1 ml 0.33% agar in CMRL 1066 medium and incubated at 37°C at 7.5% CO_2 in

air for the indicated time period. The results are shown in Table 4.

Table 4

Effect of THU on colony number obtained from $1x10^5$ blood cells cultured in agar

	Day 7			Day 10			Day 13		
Exp. No.	Contr	THUx1	THUx2	Contr	THUx1	THUx2	Contr	THUx1	THUx2
G39	0.2		0.5	9.5		30.3	728		1847
G43	9.8	29.8	56.7	215	231	753	488	694	901
G48	0	0.3	0.2	14.2	238	227	96	806	852
G52	2.8	10.2	8.8	38.5	501	535	1166	1674	1609

An increase in the number of colonies from seeded white blood cells was observed in blood taken from mice treated with THU compared to control animals. THU thus appears to increase the number of haematopoietic progenitor cells in blood.

CLAIMS:

1. A compound selected from cytidine deaminase and a functional fragment thereof for use in the regulation of cell proliferation.

2. The use of cytidine deaminase or a functional fragment thereof for the manufacture of a medicament for the regulation of cell proliferation.

3. The compound or use according to claim 1 or 2 wherein the cytidine deaminase or a functional fragment thereof is for use in combination with one or more cofactors which enhance the activity of cytidine deaminase.

4. The compound or use according to any one of claims 1 to 3 wherein haemopoiesis is inhibited.

5. The compound or use according to claim 4 wherein granulopoiesis is inhibited.

6. The compound or use according to claim 5 wherein the cell proliferation in chronic myelogen leukaemia is inhibited.

7. The compound or use according to any one of claims 3 to 6 wherein the cofactors are cytidine deaminase potentiating nucleosides or analogues thereof.

8. The compound or use according to claim 7 wherein the potentiating nucleosides or analogues thereof are selected from the group consisting of pyrimidine nucleosides or analogues thereof.

9. The compound or use according to claim 8 wherein the pyrimidine nucleosides or analogues thereof are selected

from the group consisting of thymidine, deoxycytidine, deoxyuridine and their phosphate derivatives.

10. The compound or use according to claim 9 wherein the nucleoside is thymidine.

11. The compound or use according to claim 10 wherein thymidine is present at a concentration in the range $5x10^{-3}$ to $1x10^{-6}$M.

12. A composition comprising a combination of cytidine deaminase or a functional fragment thereof and one or more cofactors which enhance the activity of cytidine deaminase for use in the regulation of cell proliferation.

13. The use of one or more cytidine deaminase potentiating nucleosides or analogues thereof for the manufacture of a medicament for the regulation of cell proliferation mediated by cytidine deaminase.

14. The use according to claim 13 wherein the potentiating nucleosides or analogues thereof are selected from the group consisting of thymidine, deoxycytidine, deoxyuridine and their phosphate derivatives.

15. The use according to claim 14 wherein thymidine and deoxycytidine are used.

16. The use according to any one of claims 13 to 15 wherein nucleosides or analogues thereof are added to give concentrations thereof of 10^{-4} to 10^{-6}M.

17. The use according to claim 16 wherein said concentrations are in the range $5x10^{-4}$ to $5x10^{-5}$M.

18. The use of one or more cytidine deaminase inhibitors in the preparation of a medicament for the regulation of cell proliferation.

19. The use according to claim 18 wherein the cell proliferation is mediated by cytidine deaminase.

20. The use of one or more cytidine deaminase inhibitors in the preparation of a medicament for the mobilization of haematopoietic stem cells to the blood.

21. The use according to any one of claims 18 to 20 wherein the inhibitor is tetrahydrouridine (THU).

22. The use according to any one of claims 18 to 21 wherein cell proliferation is stimulated in leukopenic situations such as after bone marrow transplantation or after chemotherapy.

23. A pharmaceutical composition comprising (a) cytidine deaminase or a functional fragment thereof optionally in combination with a cytidine deaminase potentiating nucleoside or analogue thereof or (b) an inhibitor of cytidine deaminase, together with at least one physiologically acceptable carrier or excipient.

24. A method of treatment of the human or non-human animal body to regulate cell proliferation, said method comprising administering to said body cytidine deaminase or a functional fragment thereof.

25. A method of treatment according to claim 24, said method comprising administering to said body in combination with the cytidine deaminase or a functional fragment thereof, one or more cofactors which enhance the activity of cytidine deaminase.

26. A method of treatment of the human or non-human animal body to regulate cell proliferation, said method comprising administering to said body one or more cytidine deaminase inhibitors.

27. Compositions containing cytidine deaminase and cofactors which enhance the activity of cytidine deaminase as a combined preparation for simultaneous, separate or sequential use in regulating cell proliferation.

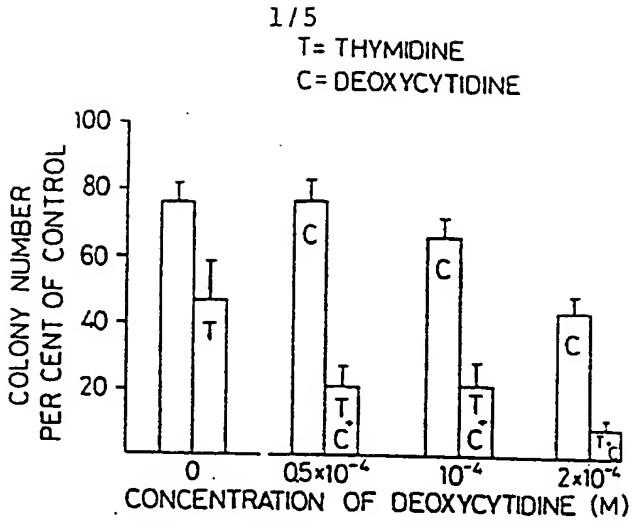

FIGURE 1

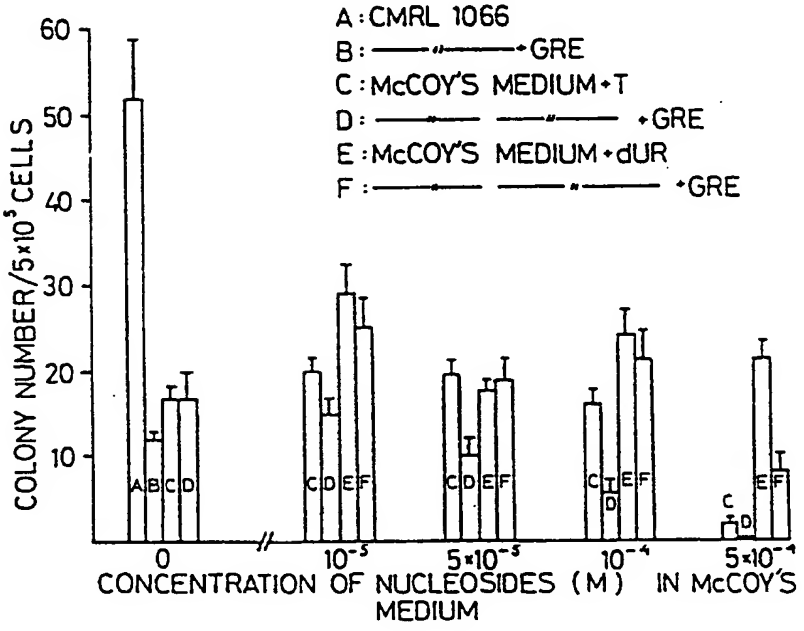

FIGURE 2

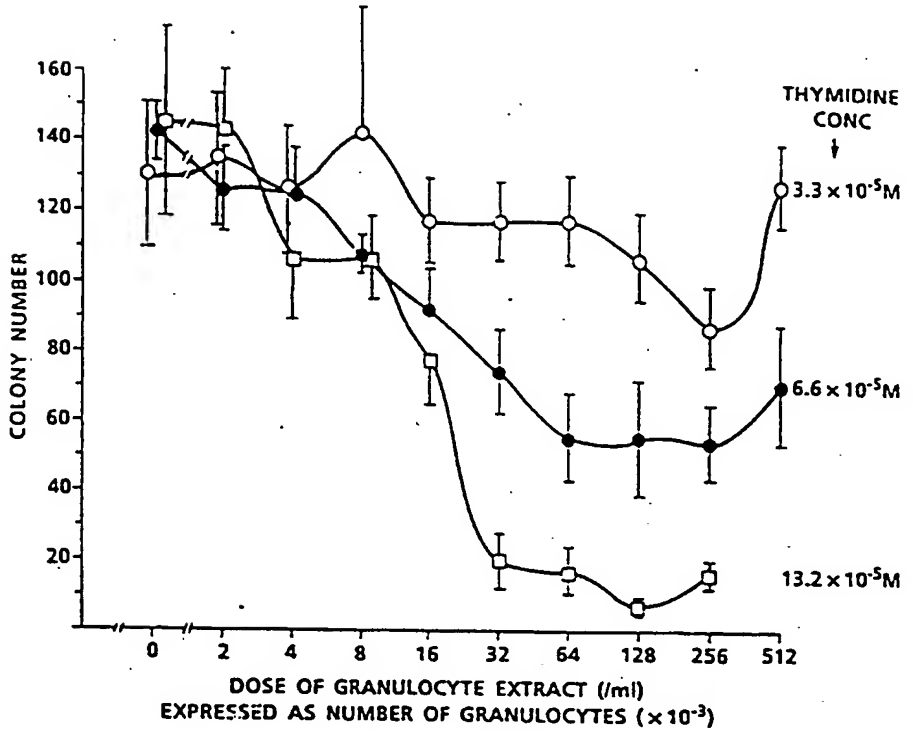

FIGURE 3

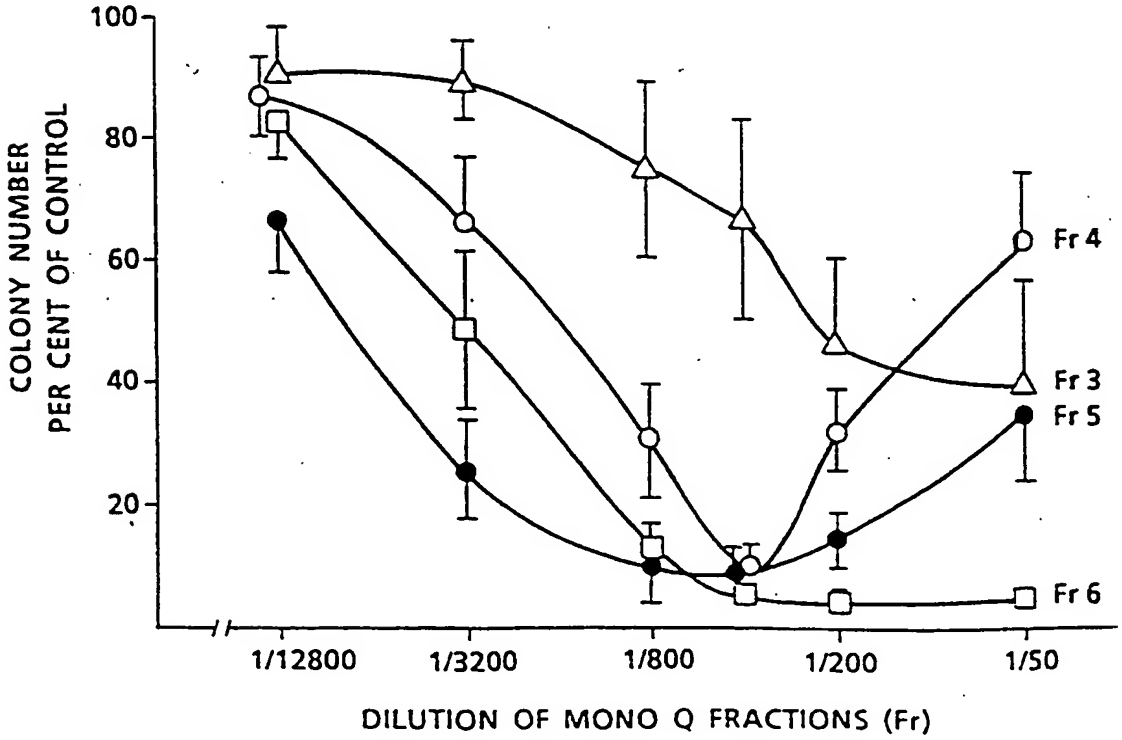

FIGURE 4

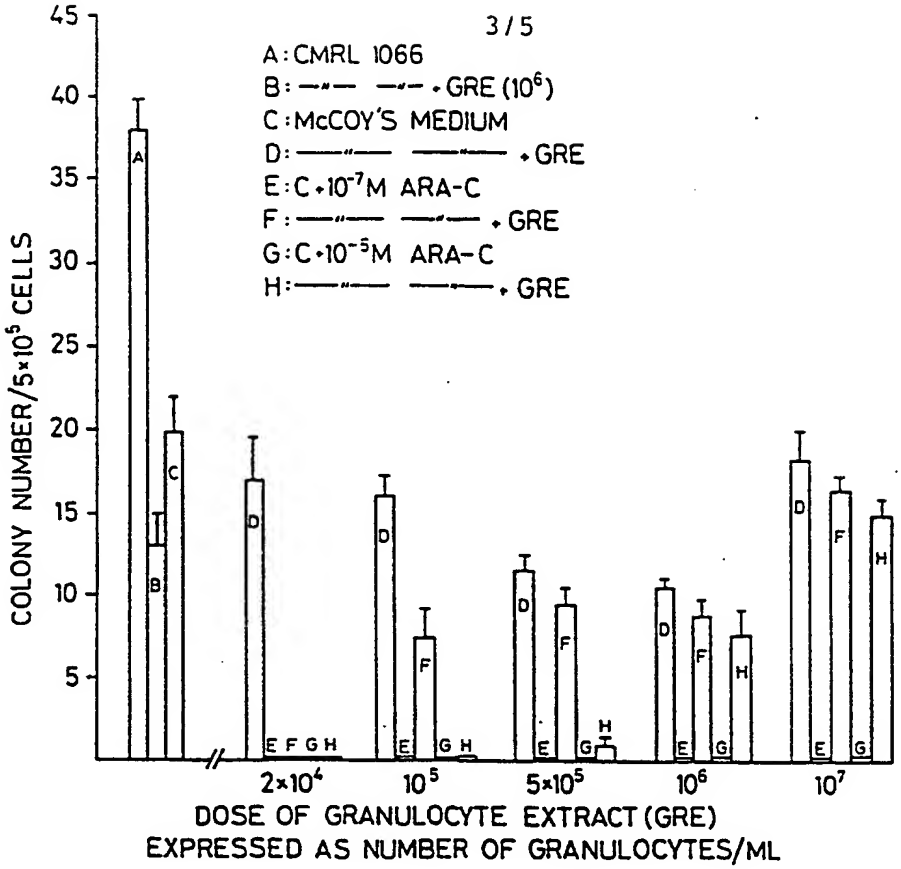

FIGURE 5

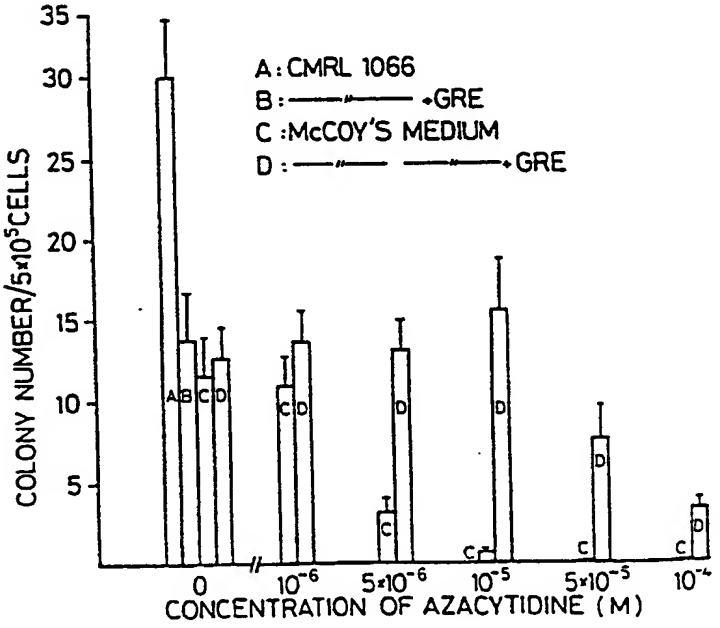

FIGURE 6

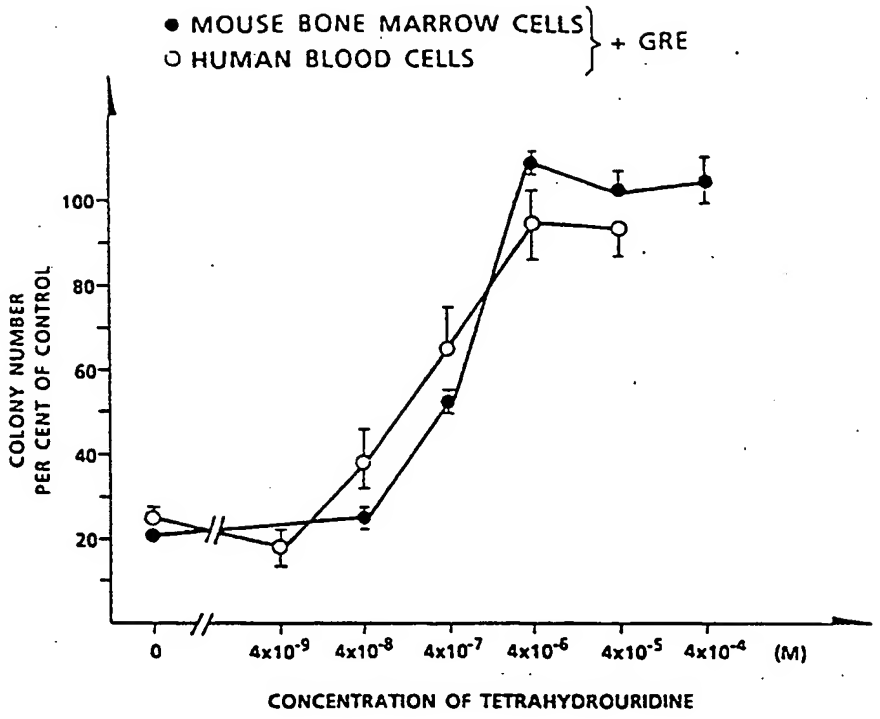

FIGURE 7

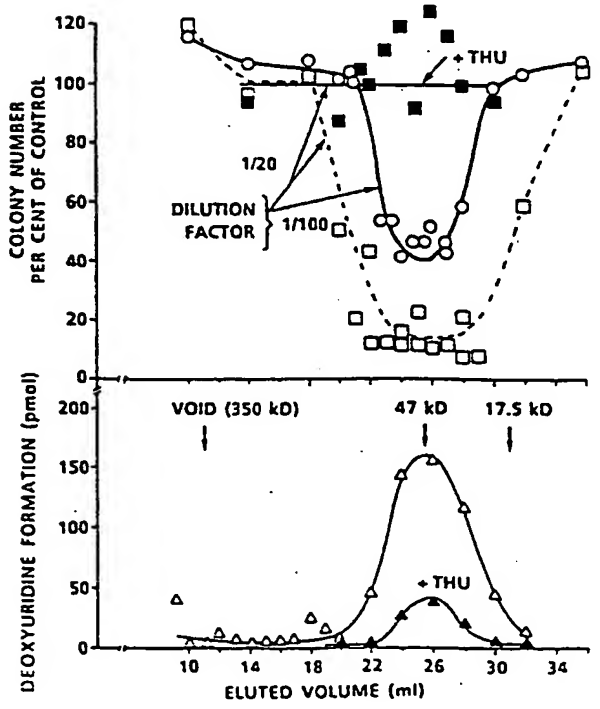

FIGURE 8

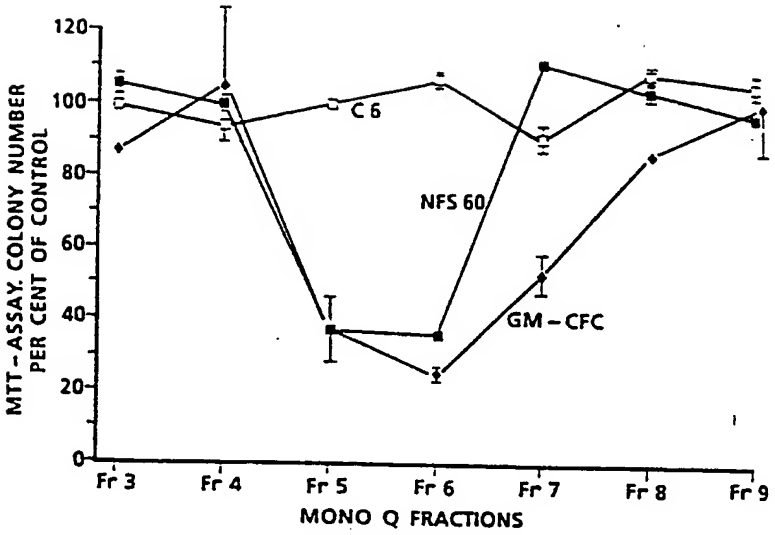

FIGURE 9

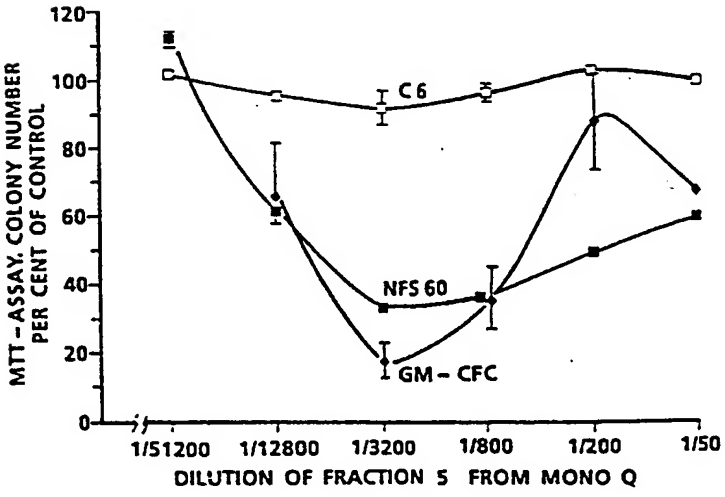

FIGURE 10

INTERNATIONAL SEARCH REPORT

Interv ›al Application No
PCT/GB 94/01190

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	EXPERIMENTAL HEMATOLOGY vol. 22, no. 2 , February 1994 pages 208 - 214 ARNE BOYUM ET AL. 'IDENTIFICATION OF CYTIDINE DEAMINASE AS INHIBITOR OF GRANULOCYTE-MACROPHAGE COLONY FORMATION.'	1-27

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 1994	14 -10- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/GB94/01190

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 24-26 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.